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ESTIMATION OF BACTERIAL DENSITIES BY MEANS OF THE "MOST PROBABLE NUMBER"*

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INTRODUCTION

THIS PAPER attempts to give a simple account of the concept of the "most probable number" (m.p.n.) of organisms in the dilution method. The concept is quite old, going back to McCrady (4) in 1915, and has been discussed by various writers from time to time, so that little of what I shall present is new. In addition, some advice is given on the planning of dilution series.

The dilution method is a means for estimating, without any direct count, the density of organisms in a liquid. It is used principally for obtaining bacterial densities in water and milk. The method consists in taking samples from the liquid, incubating each sample in a suitable culture medium, and observing whether any growth of the organism has taken place. The estimation of density is based on an ingenious application of the theory of probability to certain assumptions. For a biologist, it is more important to be clear about these assumptions than about the details of the mathematics, which are rather intricate.

ASSUMPTIONS

There are two principal assumptions. In statistical language, the first is that the organisms are distributed *randomly* throughout the liquid. This means that an organism is equally likely to be found in any part of the liquid, and that there is no tendency for pairs or groups of organisms either to cluster together or to repel one another. In practice this implies that the liquid is thoroughly mixed, and if the volume of liquid is not too great some shaking device is usually employed for this purpose.

*Paper 254 from the Department of Biostatistics.

The second assumption is that each sample from the liquid, when incubated in the culture medium, is certain to exhibit growth whenever the sample contains one or more organisms. If the culture medium is poor, or if there are factors which inhibit growth, or if the presence of more than one organism is necessary to initiate growth, the m.p.n. gives an underestimate of the true density.

MATHEMATICAL ANALYSIS

In the mathematical analysis we relate the probability that there will be no growth in a sample to the density of organisms in the original liquid. Suppose that the liquid contains V ml., the sample contains v ml., and that there are actually b organisms in the liquid. By the second assumption, there will be no growth if and only if the sample contains no organisms. We will calculate the probability that none of these b organisms is in the sample.

Consider a single organism. By the first assumption, the probability that it lies in the sample is simply the ratio of the volume of the sample to that of the liquid, i.e. v/V. The probability that it is not in the sample is therefore $(1 - v/V)$. Since there is assumed to be no kind of attraction or repulsion between organisms, these two probabilities hold for *any* organism, irrespective of the positions of the other organisms. (Strictly, this requires the additional assumption that the space occupied by an organism is negligible relative to v.) Consequently, by the multiplication theorem in probability, the probability that none of the b organisms is in the sample is

$$p = (1 - v/V)^b.$$

When v/V is small, this is closely approximated by

$$p = e^{-vb/V}$$

where e, about 2.7, is the base of natural logarithms. Finally, since b/V is the density δ of organisms per ml., we have

$$p = e^{-v\delta},$$

where p is the probability that the sample is sterile.

THE CASE OF A SINGLE DILUTION

If n samples, each of volume v, are taken, and if s of these are found to be sterile, the proportion s/n of sterile samples is an estimate of p. Hence we obtain an estimate d of the density δ by the equation

$$\frac{s}{n} = e^{-vd}.$$

This gives

$$d = -\frac{1}{v} ln\left(\frac{s}{n}\right) = -\frac{2.303}{v} \log\left(\frac{s}{n}\right) \tag{1}$$

where ln and log stand for logarithms to base e and to base 10 respectively.

The estimate d is the "most probable number" of organisms per ml. The derivation given here does not reveal why this name has been ascribed to the estimate. In fact, the concept of m.p.n. is scarcely needed for this simple case. We will, however, reexamine the analysis so as to introduce the concept, which becomes useful in the more complex situation where several dilutions are used.

If p is the probability that a sample is sterile, the probability that s out of n samples are sterile is given by the binomial distribution as

$$\frac{n!}{s!(n-s)!} p^s(1-p)^{n-s} \tag{2}$$

Since $p = e^{-v\delta}$, this expression may be written

$$= \frac{n!}{s!(n-s)!} e^{-sv\delta}(1 - e^{-v\delta})^{n-s} \tag{3}$$

If we have obtained s sterile samples out of n, this formula enables us to plot the probability of this event against the true density δ. Such curves always have a single maximum.

A curve of this type suggests a method for estimating δ which is plausible on intuitive grounds. For if we are considering two possible values of δ, it seems reasonable to prefer the one which gives a higher probability to the result that was actually observed. This argument, carried to its conclusion, leads to a choice of the value of δ for which the probability of obtaining the observed result is greatest. It is this value of δ that has been called the "most probable number" of organisms. It can be shown mathematically that this is the value of δ for which $p = s/n$. Consequently the m.p.n. is the same as the estimate previously given.

In practice, more than one dilution is usually needed. The reason is that the precision of the m.p.n. is very poor when the volume v in the sample is such that the samples are likely to be all fertile or all sterile. When all are fertile, the maximum on the probability curve (3) occurs when δ is infinite, so that the estimated density is infinite. When all are

sterile the estimated density is zero, as may also be verified from equation (1). Thus a single dilution is successful only if v happens to be chosen so that some samples are sterile and some are fertile. Such a choice of v can be made only if the density δ is known fairly closely in advance.

If we possess this knowledge, it is best to select v so that the expected number of organisms per sample lies somewhere between 1 and 2. For this choice the expected percentage of sterile samples will lie between 15% and 35%. In default of this knowledge, the practice is to use several dilutions (i.e. several different values of v) in the hope that at least one of them will give some sterile and some fertile samples.

THREE DILUTIONS

The case of three dilutions serves to illustrate the general problem. Let the suffix i indicate the dilution. For the ith dilution the volume of the sample is v_i, and s_i out of n_i samples are found to be sterile. How do we estimate δ from these results?

From equation (1) we can obtain a separate estimate for each dilution: i.e.

$$d_i = -\frac{2.303}{v_i} \log \left(\frac{s_i}{n_i}\right).$$

However, the best way to combine the three estimates d_i into a single value is not obvious. Since, as we have seen, some dilutions give very poor estimates, it is not satisfactory to take the arithmetic mean.

One solution is provided by the m.p.n. concept, which extends easily to this situation. Following the approach used in the previous section, we first write down the probability of obtaining the observed results for any hypothetical value of the true density δ. The observed results are that s_1 samples out of n_1 are sterile at the first dilution, s_2 out of n_2 at the second, and s_3 out of n_3 at the third. The probability that these three events should all happen is the product of three terms, each like expression (3) in the previous section. As before, the graph of this probability against δ shows a single maximum. The value of δ at this maximum is taken as the m.p.n.

The value of the m.p.n. cannot be written down explicitly. The equation which it satisfies is as follows.

$$s_1v_1 + s_2v_2 + s_3v_3 = \frac{(n_1 - s_1)v_1e^{-v_1d}}{1 - e^{-v_1d}} + \frac{(n_2 - s_2)v_2e^{-v_2d}}{1 - e^{-v_2d}} + \frac{(n_3 - s_3)v_3e^{-v_3d}}{1 - e^{-v_3d}}$$

Methods for solving this equation by trial and error have been given by several writers: e.g. Halvorson and Ziegler (3), Barkworth and Irwin (1)

and Finney (2). In laboratories where the numbers of samples n_i and the dilution ratios are standardized, it is convenient to have a table which gives the m.p.n. for all sets of results that are likely to occur. A table is provided in "Standard methods for the examination of water and sewage" (5), for dilution series in which 5 samples are taken at each dilution and there are three 10-fold dilutions. A more extensive table, for dilution ratios of 2, 4, and 10 and any number of levels (except two levels with a 10-fold dilution) is given by Fisher and Yates (6). This is not a table of the m.p.n., but of a different estimate which seems to be just about as precise for series of the size usually conducted in practice. This estimate is derived from the total numbers X and Y of fertile and sterile samples. The quantities $x = X/n$, $y = Y/n$ are entered in the table, from which an estimate of $\log d$ is obtained.

CRITIQUE OF THE M.P.N.

We have seen that the m.p.n. is an estimate of the density of organisms. Considered more generally, it is a *procedure for obtaining estimates*, since the same argument could be applied to other statistical problems. The only justification which I have mentioned for the procedure is that it seems intuitively reasonable. From a reading of the literature I am not certain as to the reasons which led early investigators to select this estimate, though either the intuitive approach or an appeal to a theory of inverse probability may have been responsible.

During the past 25 years the problem of making estimates from data has received much attention from statisticians. Today, most statisticians would, I believe, reject an appeal to intuition or to the theory of inverse probability as a reliable procedure for constructing estimates, since both have been found on occasion to be untrustworthy. They might also object to the name "most probable number," on the grounds that the adjective "probable" in that phrase has a different meaning from the one given to it in the theory of probability. The estimate is "most probable" only in the roundabout sense that it gives the highest probability to the observed results. But they would not reject the m.p.n. procedure itself, which has come to be regarded as a remarkably reliable tool of very wide utility. At the risk of a slight digression it is interesting to indicate the reasons for the reputation which the method has acquired.

The modern approach is to appraise any method of estimation by results. For the m.p.n. this is done, ideally, by conducting a large number of dilution series with given v's and n's, in circumstances where the true density is known. For each series the density is estimated by the m.p.n., so that we accumulate a large number of observations on the amounts by which the m.p.n. is in error. These observations can be

summarized conveniently by plotting the frequency distribution of the m.p.n. about the true density. If this frequency distribution groups very closely about the true density, we know that the estimates are usually good. Such a set of experiments would be difficult and expensive to conduct, but if we assume that the mathematical analysis which has been applied to the dilution method is valid, we can work out the frequency distribution by purely mathematical methods.

As the numbers of samples n_i become large, the frequency distribution of such an estimate (m.p.n. or other) usually tends to assume a certain limiting form—the normal distribution. An important general result has been established about these limiting distributions (7), to the effect that the limiting distribution of the m.p.n. has the smallest standard deviation that can be achieved by any method of estimation. Roughly speaking, this means that the m.p.n. gives on the average at least as precise estimates as any other method used on the same data. There is no point in seeking further for a more precise estimate. The theorem cannot be proved in general when the numbers of samples are small, but experience suggests that the m.p.n. technique is among the best methods of estimation in this case also. Consequently the m.p.n. method is now generally used in a great variety of problems of statistical estimation, though it more frequently goes by the name of the "method of maximum likelihood."

THE PLANNING OF DILUTION SERIES

In preparation for an estimation by the dilution method, three decisions must be made: (i) what range is to be covered: i.e. what are to be the highest and lowest sample volumes; (ii) what dilution factor is to be used; and (iii) how many samples should be taken for each dilution.

Specific decisions must depend on a knowledge of the limits within which the true density is likely to lie and on the precision desired in the estimate. The way in which precision is to be measured needs some comment. Suppose that the true density is thought to lie somewhere between say 2 and 400 organisms per ml. No matter where the true density should happen to be within this range, we want to plan the series so that the estimate will have a specified "precision." This might be taken to mean that the standard error of the estimated density should be say 30 organisms. But this does not seem a reasonable definition of "equal precision," because although an estimate of 360 ± 30 organisms seems satisfactorily precise, an estimate of 5 ± 30 organisms seems very imprecise. Instead, we take "equal precision" to imply that the standard error bears a constant ratio to the true density, in other words that the coefficient of variation of the estimated density is constant. A further

potent reason for adopting this concept is that in a well-designed series the m.p.n. estimates do have approximately the property that the coefficient of variation is independent of the true density. Thus in a sense we are making a virtue of necessity.

The following remarks are intended as a rough guide in the planning of dilution series. They were derived from investigations of the precision of the m.p.n.

HIGHEST AND LOWEST SAMPLE VOLUMES

These are determined by the range of densities with which we expect to have to cope. With a single dilution it was mentioned that for the best results the expected number of organisms in the sample volume v should lie between 1 and 2. It follows that in a series of dilutions the expected number of organisms in the *highest* sample volume v_H should be at least 1, otherwise there is a risk that all samples will be sterile. Similarly the expected number of organisms in the *lowest* sample volume v_L should not exceed 2, to avoid the risk that all samples will be fertile. This line of reasoning would lead to the rule that a dilution series is capable of estimating any density that lies between $1/v_H$ and $2/v_L$.

This rule is satisfactory if a substantial number of samples, say 20 or more, are being taken at each dilution. With very small numbers of samples per dilution, which are typical in certain lines of work, the rule is not quite stringent enough, in that it allows too much risk that all samples may be fertile. Suppose that we have three 10-fold dilutions, with sample volumes 0.01, 0.1 and 1 ml. This series should be able to estimate any true density between 1 and 200 organisms per ml. If, however, the density happens to be 200 per ml., so that the expected number of organisms per sample in the lowest sample volume is 2, then the probability of a sterile sample at this dilution is e^{-2}, or 0.135. The probability of a fertile sample is 0.865. If only four samples are used per dilution, the probability that all four are fertile is $(0.865)^4$, or 0.56. At the two higher concentrations, all samples are practically certain to be fertile. Thus the worker runs about a 50-50 chance that all his samples will be fertile, which usually necessitates repetition of the series. On the other hand, with 20 samples per dilution, the probability that all are fertile is $(0.865)^{20}$, or only about 0.05.

Thus in small experiments it is safer to reduce the upper density value from $2/v_L$ to $1/v_L$. In practice, we use this rule by first guessing two limits δ_L and δ_H between which we are fairly certain that the true density lies. The sample volumes are then chosen to satisfy the rules

$$v_H \geq \frac{1}{\delta_L} ; \qquad v_L \leq \frac{1}{\delta_H} .$$

For example, if we are confident that the density lies between 10 and 750 per ml., the highest sample volume should be at least 1/10, or 0.1 ml. The lowest sample volume should not be more than 1/750 ml. The three 10-fold dilutions 1/10, 1/100 and 1/1000 ml., or the four 5-fold dilutions 1/10, 1/50, 1/250 and 1/1250, would amply cover this range of densities.

THE DILUTION RATIO

As regards the selection of a dilution ratio, there are two relevant results. If the total number of samples in the whole series is kept fixed, the average precision is practically the same for any dilution ratio between 2 and 10. The advantage of a low dilution ratio, which requires more work, is that the precision is more nearly constant throughout the range of densities between $1/v_H$ and $1/v_L$. These points may be illustrated by a comparison between the dilution ratios 2 and 10, in series designed to cover the same range of densities and to use the same total number of samples, 72. The details for the two series are as follows.

Dilution ratio	No. of samples per dilution	Volumes of samples (ml.)
2	9	.01, .02, .04, .08, .16, .32, .64, 1.28
10	24	.01, .10, 1.00

The two series should cover a range of densities from $1/v_H$ to $1/v_L$, or from about 1 to 100 organisms per ml. The dilution ratio 2 requires eight dilutions, with 9 samples per dilution, whereas the dilution ratio 10 requires only 3 dilutions and allows 24 samples per dilution.

In Figure 1 the standard error of the m.p.n., expressed as a percent of the true density, is plotted against the true density (on a log scale). With both dilution ratios the standard error per cent is fairly constant for any true density between 1 and 100 organisms per ml. Outside these limits the standard error begins to rise steeply, except that with the 10-fold series, which has 24 samples per dilution, the rise is postponed until $\delta = 200$, for reasons given in the previous section. Inside the limits the standard error shows a periodic fluctuation which is noticeable with the 10-fold dilution but negligible for the 2-fold. With a 5-fold dilution (not shown), this periodic effect would be just perceptible. It is present with the 10-fold series because practically all the information is contributed by a single dilution. When the true density is about 1.5 or 15

or 150, so that one of the dilutions has about 1.5 organisms per sample, there is a trough, with peaks in the intervening densities where no sample has a density close to this value. With the 2-fold series, several dilutions contribute information and the periodic effect is smoothed out. On the whole, the 2-fold dilution gives a slightly lower standard error over the range from 1 to 100 organisms per ml., the difference being about 7 per cent. For these reasons a low dilution ratio is preferable if the extra work involved can be accomplished easily.

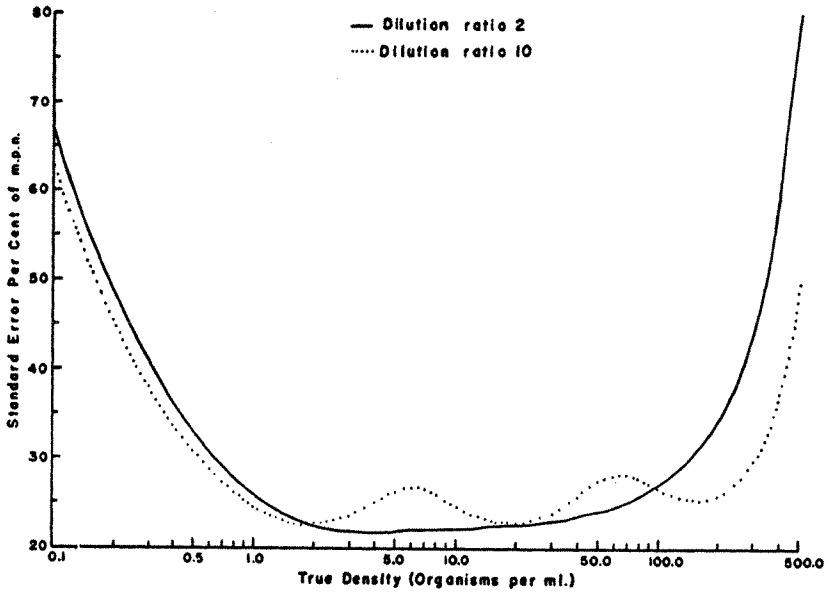

FIGURE I. COMPARISON OF DILUTION RATIOS 2 AND 10

The curves in Figure 1 were calculated by assuming that the formula which holds for the standard error in the limiting distribution, appropriate for very large samples, could be applied to this example in which the total number of samples is 72. Some unpublished work by Dr. I. J. Bross on the distribution of the m.p.n. in small samples indicates that the standard errors are higher than those obtained in this way from the limiting distribution. Further, the periodicity with the 10-fold dilution does not follow the course predicted for it. However, the two principal conclusions from Figure 1 still appear to hold in small samples, namely that the standard error is more stable with a low dilution ratio, and also tends to be slightly lower.*

*This work was carried out under contract with the Office of Naval Research.

STANDARD ERROR OF THE M.P.N.

In many types of investigation there may be only a few samples for each dilution. In this event the distribution of the estimated density d is very skew, and to attach a standard error to d is misleading. The distribution of log d is more nearly symmetrical, and it is recommended that tests of significance and the construction of confidence limits be performed from log d rather than from d. If there are n samples *per dilution* (assumed the same in all dilutions), the standard error of $\log_{10} d$ may be taken as

$$0.55 \sqrt{\frac{\log_{10} a}{n}}$$

where a is the dilution ratio. This formula can be used for any density which lies between $1/v_H$ and $1/v_L$, and for any dilution ratio of 5 or less. For a dilution ratio of 10, a more conservative factor of 0.58 is preferable to 0.55, to allow for the contingency that the estimation may have been made at a point where the standard error has one of its peaks. Thus for dilution ratio 10 the formula becomes simply $0.58/\sqrt{n}$. Note that the formula does not explicitly involve the number of dilutions used.

To test the significance of the difference between two estimated densities, made from independent series, we compute

$$\frac{\log d_1 - \log d_2}{0.55 \sqrt{\frac{\log a_1}{n_1} + \frac{\log a_2}{n_2}}}$$

and refer to the normal probability tables.

The construction of confidence limits may be illustrated by assuming that we have three 10-fold dilutions, with 5 samples per dilution. The standard error of log d is $0.58/\sqrt{5}$, or 0.259, so that the 95 per cent confidence limits for log d are (log $d \pm 0.518$). It follows that to get the upper confidence limit for d, we must multiply d by antilog (0.518) or 3.3, and to get the lower confidence limit we must divide d by 3.3.

For the common dilution ratios, 2, 4, 5, and 10, Table I shows the standard error of log d for any number of samples per dilution between 1 and 10. The table also gives the factor by which the estimated density must be multiplied and divided in order to obtain upper and lower 95 per cent confidence limits respectively. In the example presented by Fisher and Yates (6), the number of rope spore organisms per gram of potato flour was estimated to be 760. The dilution ratio was 2 and there were 5 tubes per dilution. From Table I, the factor for $n = 5$, $a = 2$ is 1.86. Hence the upper confidence limit is 760×1.86 or 1414, while the

TABLE I
STANDARD ERROR OF LOG d AND FACTOR FOR CONFIDENCE LIMITS

No. of samples per dil.	S.E.$_{(\log_{10} d)}$ Dilution ratio (a)				Factor for 95% confidence limits Dilution ratio (a)			
n	2	4	5	10	2	4	5	10
1	.301	.427	.460	.580	4.00	7.14	8.32	14.45
2	.213	.302	.325	.410	2.67	4.00	4.47	6.61
3	.174	.246	.265	.335	2.23	3.10	3.39	4.68
4	.150	.214	.230	.290	2.00	2.68	2.88	3.80
5	.135	.191	.206	.259	1.86	2.41	2.58	3.30
6	.123	.174	.188	.237	1.76	2.23	2.38	2.98
7	.114	.161	.174	.219	1.69	2.10	2.23	2.74
8	.107	.151	.163	.205	1.64	2.00	2.12	2.57
9	.100	.142	.153	.193	1.58	1.92	2.02	2.43
10	.095	.135	.145	.183	1.55	1.86	1.95	2.32

lower limit is 760/1.86 or 409. This factor clearly fulfills the same general purpose as would a standard error, if it had been appropriate to attach one to d.

The table makes it evident that the dilution method is of low precision, as is to be expected from a method that does not use direct counts. Large numbers of samples must be taken at each dilution if a really precise result is wanted. Further, the table is likely to overestimate the accuracy of the method, since it is derived on the assumption that the mathematical analysis corresponds exactly to the practical situation. With a large volume of liquid that cannot be mixed, the distribution of organisms may be far from homogeneous. The method will determine the density in that part of the liquid from which the initial sample was taken. This might be very different from the average density over the whole liquid, and this source of error could be more important than the error in the dilution method itself.

SUMMARY OF STEPS IN PLANNING

The decisions to be made involve a choice of the dilution ratio, a, the number of dilutions and the actual sample volume in each dilution, and finally the number of samples n to be used at each dilution. The steps may be set out as follows.

1. Decide on the limits δ_L and δ_H within which the true density appears certain to lie.

2. Calculate the lowest and highest sample volumes by means of the relations

$$v_H = \frac{1}{\delta_L}, \qquad v_L = \frac{1}{\delta_H}.$$

3. Select a dilution ratio. A low ratio is preferable whenever feasible.

4. The number of dilutions and the actual volumes for each dilution may now be chosen so as to satisfy the requirements that the highest sample volume must not be less than v_H and the lowest must not exceed v_L.

5. The precision to be expected for any specified number n of samples per dilution may be appraised from Table I, if the number of samples per dilution is less than 10, or from the formula for $\text{S.E.}_{(\log d)}$. Choose the number of samples in the light of the precision that is desirable and the amount of work that it is practicable to do.

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